

**PATENT**

**METHOD OF DELIVERING A PROTEIN TO POULTRY**

This is a continuation-in-part of application number 09/122,299; filed July 24, 1998.

**Background of the Invention**

**1. Field of the Invention:**

The present invention relates to poultry vaccines and, more particularly, to a novel method of delivering a protein to poultry involving spraying with a live avirulent derivative of an enteropathogenic bacterium.

**5 2. Description of Related Art:**

Contamination of poultry meat and eggs by enterobacterial human pathogens, such as *Salmonella spp.* is a well known cause of illness in humans when such contaminated products are consumed. The contamination occurs predominantly during processing of carcasses after slaughter by contact with intestinal contents that contain high levels of such enterobacteria. The enterobacteria colonize the intestinal tract, but do not normally cause disease in the poultry. In order to reduce the 10 contamination of food with enteropathogens it would thus be desirable to diminish the amount of human enteropathogenic bacteria present in the intestinal tracts of market-

age broilers. Efforts to reduce this contamination have focused on improved sanitation during production and processing (Bailey, J.S., *Poult. Sci.*, 72:1169 - 1173, 1993), but such techniques are time-consuming and expensive and are not totally effective in avoiding sporadic contamination. (See, e.g., *Food Borne Disease Outlook Annual Summary, 1982*; and *Salmonella Surveillance Annual Survey 1992*; both available from Center for Disease Control, U.S. Department of Health and Human Services, Atlanta, GA). Methods that depend upon sanitation during processing must be repeated frequently since processing equipment and personnel can be re-contaminated by each contaminated fowl that is processed. Methods that depend upon sanitation during production require constant vigilance due to the high potential for contamination in the production environment. Therefore, a simple and inexpensive method to control enteropathogenic microbes in poultry during growth would be a key improvement in reducing carcass contamination during processing.

Promsopone *et al.*, *J. Food Protect.*, 61(2): 176-180, 1998, have reported that *S. typhimurium* colonization of the intestinal tracts of poultry can be reduced by administration of an avian-specific probiotic combined with *S. typhimurium* specific antibodies. *Lactobacillus acidophilus*, *Streptococcus faecium* and *S. typhimurium*-specific antibodies were administered by spraying the chicks at one day of age followed by oral administration via the drinking water from day 1 to day 3. The chicks were challenged by oral administration of *S. typhimurium* on day 1 and significantly reduced amounts of *S. typhimurium* were recovered from the cecum and colon following probiotic-treatment at 31, 38 and 43 days. Although administration of probiotic and antibodies as early as 1 day of age may have been important in reducing colonization of the intestine by *S. typhimurium*, it is not clear from this report whether the initial spray administration of probiotic and antibodies or the more commonly used oral administration in the drinking water on days 1-3 was responsible for decreasing *S. typhimurium* colonization.

Vaccines for use in preventing diseases in poultry have been reported and some of these vaccines are specific for *Salmonella* (See, e.g. U.S. Patent No.'s 30 5,294,441, 5,389,368, 5,468,485 and 5,387,744). The methods for administration of vaccines in poultry vary, however, depending upon the target site of action of the active agent. In fact, it is commonly believed that the vaccination route should be

tailored according to the preferential site of the microorganism for localization and replication. Thus, for Newcastle disease and infectious bronchitis viruses which multiply in the respiratory route, the vaccination methods of choice would be by eye drop into the eye, nasal passage and respiratory system of the chick or by the spray route. (Giambrone, *World Poultry- Misset 13:19-23, 1997*). Since many of the more important diseases of poultry occur in the respiratory tract, studies reporting on administration of spray vaccination for these diseases have used spray administration because an aerosol or spray is easily inhaled by the bird and thereby contacts the mucosal surfaces of the upper respiratory tract. Administration of vaccines for non-respiratory diseases, such as diseases of the tissues, circulatory system or gut, is usually by subcutaneous injection, or by oral administration, either by inoculation or by application in drinking water.

References disclosing the use of the spray administration of vaccines to poultry have almost exclusively been directed to immunizing against viral agents that invade through the upper respiratory tract such as, for example, to prevent Newcastle disease, avian encephalomyelitis, Marek's disease, laryngotracheitis, infectious bronchitis and the like. The reasons for this state of affairs are likely based on the commonly accepted knowledge that, in mammals, aerosol immunization has the potential to elicit both mucosal and systemic immunity, the balance between the two being manipulated by the aerodynamic particle size of the aerosol. Antigen delivered in aerosols with particle size  $>5 \mu\text{m}$  impinges on the mucosa of the upper respiratory tract, is taken up either directly, or delivered to the local lymphatic tissue and initiates mucosal immunity. Aerosols  $<5 \mu\text{m}$  deposit deep in the lung where antigen is taken up by lung-associated lymphoid tissue and transported by the draining lymphatics to the bloodstream and thus to the spleen, resulting in both a local and a systemic immune response. (See, Dertzbaugh, M. T., *et al.*, Chap. 52, pp.839 - 849, at p. 846, in *Mucosal Immunology, 2nd Ed.*, P. L. Ogra *et al.*, Eds., Academic Press, San Diego (1999)).

Bacterial vaccines, in particular live attenuated mutants derived from highly virulent bacterial parent strains, have also been used in poultry (Roland, K. *et al.*, Efficacy of *Salmonella typhimurium* vaccine strains expressing *Escherichia coli* 078 lipopolysaccharide to protect against *E. coli* challenge in chickens, Abstract of a

presentation at Conf. Of Res. Workers in Animal Diseases, Chicago, IL, Nov. 10, 1997). Derivation of the attenuated mutant strain from a highly virulent parent increases the likelihood that the attenuated mutant will not only colonize the intestinal tract but also colonize the gut associated lymphoid tissue (GALT) and elicit protective immunity. (See, e.g., Curtiss III *et al.*, in *Colonization Control of Human Bacterial Enteropathogens in Poultry*, Blankenship *et al.*, eds, Academic Press, Inc., New York, 1991, 169-198). In contrast, bacteria that colonize the intestine but do not invade and colonize the GALT may not elicit an immune reaction. For example, studies in mice have revealed that lipopolysaccharide (LPS) O-antigen repeats on the surface of *S. typhimurium* are important not only to withstand nonspecific host defense mechanisms (*Microbial Toxins*, Vol. V, Roantree *et al.*, eds., Academic Press, New York, 1971), but also for effective invasion through the mucin and glycocalyx covering the intestinal tract. As a consequence, rough mutants lacking LPS O antigens, when given orally, are unable to invade and colonize the GALT (See, e.g. 10 Curtiss *et al.*, 1991, *supra*).  
15 Curtiss *et al.*, 1991, *supra*).

Some references have reported on the administration of bacterial vaccines to poultry by oral or subcutaneous injection. For example, one commercial vaccine to prevent paratyphoid in pigeons comprises killed *S. typhimurium* administered by subcutaneous injection (Vetafarm Paratyphoid Vaccine, Vetafarm Pty. Ltd., Wagga 20 Wagga, Australia). In addition, Curtiss *et al.*, 1991, *supra*, report the use of an avirulent derivative of a pathogenic *Salmonella* as an orally administered vaccine in chicks.

Spray vaccination has also been reported for bacterial vaccines that cause respiratory diseases. Hertman *et al.* report on oral and aerosol administration of a 25 *Pasteurella multocida* vaccine to chickens and turkeys to prevent fowl cholera, which is a respiratory tract disease (U.S. Patent No. 4,169,886). Ley *et al.* report on eye-drop and aerosol administration of a vaccine containing live *Mycoplasma gallisepticum*, which produces a respiratory tract disease (Ley *et al.*, *Avian Diseases* 41:187-194, 1997). A commercially available vaccine recommends administration of 30 a vaccine containing an avirulent strain of *E. coli* serotype O78 to immunize against the respiratory disease caused by the wild-type parent (see Product Bulletin for GARAVAX® -T, Schering-Plough Animal Health Corp., Omaha, NE). The use of an

aerosol administration for all of these vaccines would have been selected because the underlying disease for which the poultry were being vaccinated involved infection of the respiratory tract.

Another reference reported that a vaccine containing a strain of the  
5 nonpathogenic *E. coli* K-12 lacking O-antigen could be administered as an aerosol (U.S. Patent No. 4,404,186). Nevertheless, the K-12 strain is a laboratory-adapted strain and is not an enteropathogen and because this microbe has no ability to invade and colonize the gut associated lymphoid tissue, it is likely that any immunity elicited by this vaccine would have been due to immunization through the respiratory route.

10 Localized spraying of bacterial vaccines such as by nasal spraying or ocular spraying had been suggested in some references (for example, see U.S. Patent No. 5,294,441). Nevertheless, none of this earlier work suggested the use of whole body spray administration of enteropathogenic bacterial vaccines.

Therefore, while spray-administered vaccines have been reported to be useful  
15 in controlling respiratory diseases in poultry, whole-body spray administration has not been suggested for vaccines in poultry for the control of human pathogens that are often present in and transmitted by poultry, but which are not the causative agents for respiratory disease in poultry.

Modern commercial poultry practice sometimes requires the delivery of  
20 proteins to the birds, other than antigenic proteins for the purpose of eliciting an immune response. For example, growth factors, immunoregulatory proteins and peptides, and other biologically active proteins and peptides are often delivered to birds. The delivery of these proteins is most commonly done by inclusion in the feed or drinking water, but these methods are useless for newly hatched chicks, since they  
25 do not eat or drink for up to several days after hatching. In addition, the inclusion of labile proteins in drinking water or feed, can often result in the inactivation of a significant portion of the active molecules due to microbial activity, thermal degradation, or oxidation.

Accordingly, it would be desirable to provide a method that could be used to  
30 deliver selected proteins to domestic poultry in a manner that would be easy and inexpensive to administer under normal commercial poultry production conditions,

and which would be effective for delivering the proteins to newly hatched chicks in an effective manner and without individual handling.

Summary of the Invention:

5       In accordance with the present invention, it has been discovered that a protein can be administered to domestic birds by whole-body spraying of the birds with an effective amount of a live avirulent derivative of an enteropathogenic bacterium that includes a recombinant gene that encodes for the expression of the protein. The enteropathogenic bacterium that serves as the carrier organism for the protein may  
10 also be referred to herein as a "vaccine" even though the purpose of the delivered protein or peptide may or may not be to elicit an immune response from the recipient. While virtually any protein can be delivered by this method, whole-body spray administration is surprisingly effective for proteins that elicit an immune response from the host bird, and also for proteins that have some other desirable property. The  
15 carrier organism for the delivery of the protein is a live avirulent derivative of an enteropathogenic bacterium. Such enteropathogenic bacteria are preferably *Salmonella* species, and it is preferred that the enteropathogenic bacterium is other than one that causes respiratory disease in birds.

The effective dose, which results in colonization by the bacterium and  
20 expression of the protein, is unexpectedly low and is roughly comparable to a dose that is effective by the oral route of administration, such as administration in the drinking water. Typically, doses for administration of the live avirulent derivative of the enteropathogenic bacterium of the present invention are from about  $10^5$  to about  $10^8$  colony forming units.

25       The spray route of administration of the protein is applicable to birds, such as chickens, at any age at which they are susceptible to the beneficial effects of the delivered protein, but is especially applicable to birds that are of an age of 3 weeks or less, and, preferably, to birds of less than 1 day of age.

In some embodiments, the spray-administration can be followed by  
30 administration of the vaccine in at least one booster dose. Preferably such a booster dose can be administered orally by drinking water or by spray at about 14 days after administration by spray.

Preferably, the spray is a coarse spray of droplets having diameters in the range of from about 50 microns to about 150 microns.

In another embodiment, the invention is directed to a domestic bird to which a protein has been delivered by the method described above.

5 Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of a new method to deliver proteins to domestic poultry in a manner that is easy and inexpensive to administer under normal commercial poultry production conditions, and which is effective for delivering the proteins to newly hatched chicks in an effective manner and without individual  
10 handling.

Brief Description of the Drawings:

Figure 1 shows the recovery of *S. typhimurium*  $\chi$ 3985 vaccine strain from the spleen and bursal tissues, feces and cecal contents of white leghorn chicks 7 days after receiving (a)  $10^5$  CFU, (b)  $10^7$  CFU, or (c)  $10^9$  CFU of  $\chi$ 3985 by coarse spray or by direct oral methods of delivery on day of hatch;

Figure 2 shows the recovery of *S. typhimurium*  $\chi$ 3985 vaccine strain from the spleen and bursal tissues, feces and cecal contents of white leghorn chicks 20 days after receiving (a)  $10^5$  CFU, (b)  $10^7$  CFU, or (c)  $10^9$  CFU of  $\chi$ 3985 by coarse spray or by direct oral methods of delivery at days 1 and 14; and

Figure 3 shows the serum IgM, IgA and IgG responses at 20 days of age as detected by using purified *S. typhimurium* LPS in white leghorn chickens immunized and boosted with (a)  $10^5$  CFU, (b)  $10^7$  CFU, or (c)  $10^9$  CFU of *S. typhimurium*  $\chi$ 3985 by coarse spray or by direct oral methods of delivery at days 1 and 14.

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Description of the Preferred Embodiments:

The present invention is based upon the discovery that desirable proteins and/or peptides can be delivered to domestic birds by the whole-body spray administration of live avirulent derivatives of enteropathogenic bacteria that encode 30 for the expression of the proteins and/or peptides.

The whole-body spray administration method of the present invention allows delivery of the vaccine, protein, or immunogenic compositions to the gastrointestinal

tract of the poultry. Spray administration or spray vaccination as used herein is intended to mean the delivery of droplets of a liquid comprising a live avirulent derivative of an enteropathogenic bacterium that includes a recombinant gene that encodes for the expression of a protein. Whole-body spray administration is intended  
5 to mean the delivery of such droplets to a large portion of the entire body of the poultry. This is in contradistinction to a localized spray administration such as is by intranasal spraying in humans in which administration is to only a specific, small, localized target area. The whole-body spray approach for administering the carrier organism of the present invention indiscriminately delivers the microbe to a large  
10 portion of the body surface of the poultry constituting that portion of the entire body surface that is accessible to the spray device (see for example, U.S. patents 4,316,464 and 4,449,968, which are incorporated by reference). Such whole-body spray administration of the organisms of the present invention is particularly applicable for administration to large numbers of poultry at the same time.

15       Spray administration in the present invention preferably involves delivery of a coarse spray containing the vaccine or immunogenic composition to the poultry. Although not wishing to be bound by a particular theory, it is believed that the administration of a vaccine or immunogenic composition as a coarse spray allows the spray droplets to contact the body surface while minimizing the amount of the vaccine  
20 that is inhaled into the lower respiratory system. This is to be distinguished from a spray of very fine droplets or mist, such as is commonly referred to as an aerosol in which droplets have a diameter of less than about 40 microns. Unlike the aerosol sprays, the coarse spray of the present invention is believed not to be deeply inhaled which assists in avoiding the development of respiratory infections seen with some  
25 spray vaccination (See for example, U.S. Patent No. 4,449,968; Clarke et al, *Austr. Vet. J.* 56:424-428, 1980). A coarse spray as used herein is intended to mean a spray that is composed of liquid droplets having a diameter sufficient to substantially prevent the inhalation of the droplets into the lower respiratory system of the bird, but still causing the liquid droplets to contact the body surface of the bird. The  
30 consistency of such a coarse spray has been referred to as "misty rain", and it is preferred that the spray have less than about 1% of the droplets in a size range of less than about 12 microns. Preferably, the coarse spray is composed of droplets having a

mean diameter of from about 40 to about 400 microns; more preferably from about 40 microns to about 200 microns, even more preferably from about 50 to about 150 microns and most preferably from about 50 to about 100 microns. Alternatively the coarse spray can have about 80% of droplets in a range of from about 90 to about 190 microns.

The type of spray vaccination equipment that is used for the administration of the vaccine is not critical and almost any type of spray vaccination equipment capable of dispensing a coarse spray can be used (see for example, U.S. Patent Nos. 4,316,464, 4,449,968, 4,674 and 5,312,353).

The spray administration of the present invention delivers a vaccine comprising a live avirulent derivative of an enteropathogenic bacterium. The vaccine microbe is an enteropathogen that is capable of colonizing the intestinal tract and gut associated lymphoid tissues (GALT) of the poultry. Such microbes serve as the immunogenic component of the vaccine or immunogenic composition and include enteropathogenic bacteria such as *Escherichia*, *Klebsiella*, *Proteus*, *Yersinia*, and *Erwinia*, *Salmonella*, *Salmonella-Escherichia* hybrids, *Shigella*, *Campylobacter*, *Providencia*, *Morganella*, *Hafnia*, *Serratia*, *Edwardsiella*, *Enterobacter* and *Citrobacter*. In particular, *Salmonella*, *Escherichia* and *Salmonella-Escherichia* hybrids are useful in the present invention, including, preferably, *E. coli* and *Salmonella* such as *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi*, *Salmonella enteritidis*, *Salmonella dublin*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella arizona*, *Salmonella enteriditis*, *Salmonella heidelberg*, *Salmonella anatum*, *Salmonella hadar*, *Salmonella agona*, *Salmonella montevideo*, *Salmonella kentucky*, *Salmonella infantis*, *Salmonella schwarzengrund*, *Salmonella saintpaul*, *Salmonella brandenburg*, *Salmonella istanbul*, *Salmonella cubana*, *Salmonella bredeney*, *Salmonella braenderup*, *Salmonella livingstone*, *Salmonella berta*, *Salmonella california*, *Salmonella senftenberg*, *Salmonella mbandaka* and *Salmonella choleraesuis*.

The avirulent derivative of an enteropathogenic bacterium can also serve as a carrier bacterium to deliver selected antigens to the GALT, gastrointestinal tract, and internal organs. Such carrier bacteria can contain and express a recombinant gene from a pathogenic organism, for example, so that antibodies and/or cellular immunity

will be elicited against the antigenic gene product normally produced by the pathogenic organism. It is thus possible to use the avirulent derivative of an enteropathogenic bacteria, administered by spray, to deliver antigens to a wide variety of microbes and to elicit an immune response in the poultry against microbes that

5 need not necessarily be able to colonize the gastrointestinal (GI) tract.

The avirulent microbes can additionally be used as vectors for the synthesis of various proteins in the poultry. Because the avirulent microbes of this invention are able to traverse the GALT after spray administration and enter into the gastrointestinal tract of the poultry, the microbes can be used to make and deliver heterologous gene

10 products such as, for example, growth factors or immunoregulatory products or substances that stimulate or suppress various physiological functions. Such microbes contain and express a recombinant gene that encodes the desired protein.

The terms enteropathogenic bacterium or enteropathogenic bacteria are intended to mean microbes that are capable of colonizing the intestinal tract and the

15 gut associated lymphoid system of the poultry. As used herein, "colonizing", means the enteropathogenic bacteria are able to attach to, invade and persist in one or more of the following tissues in the vaccinated bird: gut associated lymphoid tissue (GALT), bronchus associated lymphoid tissue (BALT), lung, spleen, liver, bursa of Fabricius, and ceca. As used herein, "pathogen" is intended to mean a microbe that is

20 capable of causing disease symptoms or impairing normal physiological functioning. The vaccines of the present invention contain avirulent derivatives of an enteropathogenic strain of bacteria. By derivative or derived strain, reference is made to a strain that has been genetically modified from its parent from which it is descended. By pathogenic it is meant that the microbe is capable of causing disease

25 or impairing normal physiological functioning.

Reference to avirulence is intended to mean that a particular virulent microbe strain has been modified so that it is incapable of inducing a full suite of symptoms of the disease state that is normally associated with its virulent pathogenic counterpart. Thus, avirulence includes a state of diminished virulence or ability to produce disease

30 conditions and the avirulent microorganisms are not necessarily completely absent of any ability to impair normal physiological functioning of the host. In addition, an avirulent microbe is not necessarily incapable of ever functioning as a pathogen, but

the particular microbe being used is avirulent with respect to the particular individual being treated. Preferably, the enteropathogenic bacterium from which the avirulent microbe is derived is pathogenic at least to day-of-hatch birds.

Live avirulent derivatives of enteropathogenic bacteria that have been found to  
5 be useful in the method of the present invention include the following:

Avirulent derivatives as described in U.S. Patent No. 4,888,170, where the  
derivatives are of a pathogenic microbe of the genus *Salmonella* or which is a  
*Salmonella-Escherichia* hybrid that express a recombinant gene derived from a  
pathogen of a vertebrate of the species *Streptococcus mutans* to produce an antigen  
10 capable of inducing an immune response in the vertebrate against the pathogen.

Preferred strains of such derivatives are *S. typhimurium* χ3115 (ATCC 39961), and  
χ3137 (ATCC 39962);

Live avirulent bacterial cells as described in U.S. Patent No. 5,424,065, which  
are an avirulent *Salmonella* which contain a mutation in the *phoP* gene, wherein the  
15 avirulent *Salmonella* are unable to cause *Salmonella*-based disease symptoms and are  
able to colonize in lymphoid tissue for a sufficient time to induce antibody and  
cellular immunity, and wherein the strain retains the properties of avirulence and  
immunogenicity of a *Salmonella* strain selected from the group consisting of ATCC  
53864, ATCC 53865, and ATCC 53866;

20 Live avirulent derivatives as described in U.S. Patent No. 5,672,345, where  
the strains are derivatives of a pathogenic strain of bacteria characterized by:

a) a lack of a functioning native chromosomal gene encoding a first enzyme  
which is a β-aspartic semialdehyde dehydrogenase (Asd);

b) the presence of a first recombinant gene encoding a second Asd enzyme  
25 wherein the first recombinant gene cannot recombine to replace the defective  
chromosomal gene;

c) the presence of a second recombinant gene encoding a desired polypeptide;  
and

d) physical linkage between the first recombinant gene and the second  
30 recombinant gene, wherein loss of the first recombinant gene causes the bacteria to  
lyse when in an environment which requires expression of the first recombinant gene  
for cell survival.

Preferred strains of such bacteria as are described in U.S. Patent 5,672,345 are provided by  $\chi$ 6097 (ATCC 67537),  $\chi$ 3520 (ATCC 53681),  $\chi$ 4072 (ATCC 67538),  $\chi$ 3008 (ATCC 53680),  $\chi$ 2108 (ATCC 53678), and  $\chi$ 6097 (ATCC 67813);

A live avirulent *Salmonella*, as described in U.S. Patent No. 5,387,744, having 5 a mutation in a *cdt* gene, where the *Salmonella* has the phenotype of failure to colonize deep tissue of *Salmonella* deposit strain ATCC no. 55113. Preferred strains of these bacteria are  $\chi$ 3958 (ATCC 55110),  $\chi$ 4323 (ATCC 55115),  $\chi$ 4346 (ATCC 55113),  $\chi$ 3940 (ATCC 55119), and  $\chi$ 4073 (ATCC 55118);

Live avirulent *Salmonella choleraesuis* as described in U.S. Patent No. 10 5,468,485, which strains are obtained from a pathogenic strain of *S. choleraesuis*, and where the avirulent *S. choleraesuis* has been made avirulent by an inactivating mutation in a *cya* gene and inactivating mutations in the *crp* and *cdt* genes. A preferred strain of such *S. choleraesuis* is  $\chi$ 3781 (ATCC 67923); and

15 Live avirulent *Salmonella typhi* as described in U.S. Patent No. 5,294,441, where the avirulent *S. typhi* are obtained from a pathogenic *S. typhi* strain and are made avirulent by an inactivating mutation in the structural *cya* gene and an inactivating mutation in the structural *crp* gene. Useful strains of the type described in U.S. Patent No. 5,294,441 are  $\chi$ 3927 (ATCC 55117) and  $\chi$ 4323 (ATCC 55115), which have  $\Delta cya$ -12/ $\Delta crp$ -11 mutations.

20 When the subject method of coarse spray administration is used to deliver a protein, to a domestic bird, the terms "deliver a protein" are intended to include the initiation of colonization by the carrier bacterium of at least one of gut-associated lymphoid tissue (GALT), bronchus associated lymphoid tissue (BALT), lung, spleen, liver, bursa of Fabricius, or ceca of the domestic bird, and preferably the colonization 25 of at least one of the tissues selected from lung, spleen, liver, bursa of Fabricius, or ceca. It is more preferred that, upon colonization, the carrier bacterium then expresses the recombinant gene to provide the protein it encodes.

The protein that is delivered by the carrier organism can be a protein that acts 30 as an antigen to elicit an immune response, as described in detail herein, or it can be a growth factor, regulatory protein, or any other protein that it is desirable to introduce into the bird. When the terms "gene product", or "protein" are used herein in the context of their delivery to a bird by the subject method of administration, it is to be

understood that these terms can pertain to a single gene product, or protein, but the terms can include two or more genes and/or proteins, and, in fact, can include a set of genes that encode enzymes necessary for the synthesis of another desirable protein or non-protein product.

5 By way of example, recombinant carrier bacterium that express several genes are described by Roland *et al.*, in *Avian Diseases*, 43:429 - 442 (1999), as being live recombinant *Salmonella* that express an O-antigen of an avian pathogenic gram negative (AP<sub>G-N</sub>) microbe due to the stable integration into the *Salmonella* chromosome of the *rfb/rfc* gene cluster and the *rfc* gene of the AP<sub>G-N</sub> microbe. The 10 *Salmonella* host has a mutation in the *Salmonella rfb* gene cluster or in the *Salmonella rfc* gene which inactivates the expression of *Salmonella* O-antigen, wherein the recombinant *Salmonella* strain is an attenuated (*i.e.*, avirulent) mutant of a virulent *Salmonella* strain. These recombinant *Salmonella* can be made to express the *E. coli* LPS O-antigens of *E. coli* strains O1, O2, O35 and O78.

15 One or more genes encoding immunoregulatory proteins or peptides may be recombinantly introduced into the avirulent microbes such that the microbes taking up residence in the appropriate immunocompetent tissue are capable of expressing the recombinant product to suppress, augment or modify the immune response in the host. Examples of immunoregulatory molecules include but are not limited to: colony 20 stimulating factors (macrophage, granulocyte, or mixed), macrophage chemotoxin, macrophage inhibition factor, leukocyte, inhibitory factors, lymphotoxins, blastogenic factor, interferon, and interleukins.

One or more genes encoding growth factors can be recombinantly introduced into the avirulent microbes, so that when the microbes take up residence in the 25 appropriate immunocompetent tissue, they are capable of expressing the recombinant product to suppress, augment or modify growth-related processes in the host. It is preferred that the growth factor that is introduced into the host bird is one that is active in the growth related processes of the host.

When the protein(s) that are delivered to the domestic bird are antigens and act 30 as vaccines, they elicit an immunological response in the bird. An immunological response to a composition or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest.

Usually, such a response consists of the subject producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included in the composition or vaccine of interest.

By vaccine is meant an agent used to stimulate the immune system of an individual so that protection is provided against an antigen not recognized as a self-antigen by the immune system. Immunization refers to the process of inducing a continuing high level of antibody and/or cellular immune response in which T-lymphocytes can either kill the invading microbe and/or activate other cells (e.g., phagocytes) to do so in an individual, which is directed against a microbe or antigen to which the organism has been previously exposed. The phrase "immune system" is intended to refer to the anatomical features and mechanisms by which an individual produces antibodies against an antigenic material that invades the cells of the individual or the extra-cellular fluid of the individual and is also intended to include cellular immune responses. In the case of antibody production, the antibody so produced can belong to any of the immunological classes, such as immunoglobulins A, D, E, G or M. Of particular interest are vaccines which stimulate production of immunoglobulin A (IgA) since this is the principle immunoglobulin produced by the secretory system of warm-blooded animals, although vaccines of the invention are not limited to those which stimulate IgA production. For example, vaccines of the nature described herein are likely to produce a broad range of other immune responses in addition to IgA formation, for example cellular and humoral immunity. Immune responses to antigens are well studied and widely reported. A survey of immunology is provided in Elgert, Klaus D., *Immunology*, Wiley Liss, Inc., (1996); Stites *et al.*, *Basic & Clinical Immunology*; 7th Ed., Appleton & Lange, (1991) the entirety of which are incorporated herein by reference.

An individual treated with a vaccine of the present invention is intended to mean one of a species of birds, including domestic birds, particularly those of agricultural importance. Domestic birds or poultry as used herein include any of a variety of domesticated avian species or individuals of that species, such as chickens, turkeys, ducks, geese, pigeons, guineas, ostriches, emus, and the like and, in particular, those domesticated avian species or individuals kept for the production of eggs or meat.

The vaccine can be prepared by growing the vaccine strain in suitable growth media and then used as is or formed into a vaccine composition by combining the growing culture, or the cells therefrom, with a suitable diluent. Suitable diluents are preferably liquids and are more preferably a liquid that does not adversely effect the stability and vitality of the vaccine culture and which has a viscosity similar to water so that it will easily form droplets of a coarse spray. The diluent is preferably free of chlorine, antibiotics, antimicrobials, or any other agent that may be harmful to the live vaccine organisms. Vaccine should be dispersible in the diluent so that no solid lumps or chunks of vaccine remain and the diluent should be at a temperature that is not harmful to the live vaccine microbes. Examples of suitable diluents include water, distilled water, de-ionized water, skim milk, water containing Marek's vaccine stabilizer, buffered saline with gelatin, and similar compositions that are well-known to persons of skill in the art. The vaccine is preferably introduced into the diluent while the diluent is at a temperature of approximately room temperature or cooler, more preferably from about 34°C to about 15°C.

In one embodiment, vaccine is prepared from *S. typhimurium* UK-1 *AcyA Acrp* χ3985 (ATCC 68166). As used herein, this vaccine may be referred to as χ3985, or as Chi3985, or as χ3985, U.S.D.A. Product Code 19C1.01. The vaccine strain can be freshly prepared as described above, or may be recovered from a culture stored, for example, as a freeze-dried culture, in a frozen form (for example, as -70°C. working seed stock), or otherwise. An inoculum from such culture is then grown to a late log-phase culture in Luria broth in 37°C. By way of example, a -70°C seed stock can be used to inoculate 50 ml of Luria broth in a 250 ml sterile flask covered loosely with foil. The flask is incubated as a static culture at 37°C overnight. After about 12 - 24 hr., 50 ml of the static overnight culture is pipetted into 450 ml of prewarmed Luria broth in a 1 L nonbaffled flask at 37°C and placed in a New Brunswick incubator shaker at 150 rpm. After the culture reaches OD<sub>600</sub> ≥ 1.0, cells are pelleted by centrifugation (4400 rpm, 15 min in a Centra MP4 centrifuge, IEC swinging bucket 3224 rotor) at room temperature. Cells are resuspended in 40 ml of room temperature buffered saline with gelatin (BSG). The titer of the vaccine composition can be determined by serially diluting the cell suspension 10-fold in BSG and spreading 100 µl of 10<sup>-6</sup> and 10<sup>-7</sup> dilutions onto MacConkey agar + 1% maltose for plating. The titer

of the vaccine strain is then determined by counting colonies that develop upon incubation of the plates. The titer is expressed in terms of colony forming units of the vaccine microbe (CFU) per unit volume of the vaccine composition.

- Vaccine for application to poultry is prepared as described above and the
- 5 culture is diluted to the desired dose density, or titer, in a suitable diluent. The buffer of the diluent, if used, is adjusted to match the pH and ionic strength required to maintain the stability and vitality of the vaccine strain. The vaccine is then ready for loading into the sprayer and for administration to the poultry.

Spray administration can also be performed in a manner to deliver a particular

10 dosage per bird. One technique that can be used to deliver an accurate vaccine dosage is to spray birds in an enclosed space for a calculated period of time at a known volumetric delivery rate. By knowing the number of birds to be vaccinated, the desired dosage of the vaccine per bird, the titer of the vaccine and the delivery rate of the spray equipment, one skilled in the art can easily calculate the spraying time

15 required to deliver the required dosage per bird. Furthermore, some models of commercially available spray equipment allow pre-selection of the volume of liquid to be delivered to a known number of birds.

The vaccine or immunogenic composition of the present invention is administered in an effective dose or an effective amount. As used herein an effective

20 amount is that quantity of vaccine or immunogenic composition which is sufficient to elicit an immune response against a target microbe or antigen for which the poultry is being vaccinated. Such immune response will involve the production of antibodies and/or cellular immunity. In one significant aspect of the present invention, the vaccine or immunogenic composition can be administered at a dose roughly equal to

25 the dose effective upon oral administration, for example by administration in the drinking water. It is preferred that the dose that is administered in the form of a coarse spray is from about  $10^4$  to about  $10^8$  colony forming units of the live avirulent derivative of a pathogenic bacterium; more preferred is a dose of from about  $10^5$  to about  $10^7$  colony forming units; and even more preferred is a dose of at least about  $1 \times 10^6$  colony forming units of the live avirulent derivative of a pathogenic bacterium.

Preferably the spray administration is given to birds when they are less than one day old, *i.e.* on the day of hatch. It is often also desirable to administer one or

more booster applications of the vaccine some time after the initial spray administration. Such booster applications can be administered at any time during the bird's life at which the bird is susceptible to the beneficial effects of the vaccine. Preferably, such booster applications are applied between 5 and 21 days of age, more 5 preferably between 6 and 15 days of age and still more preferably between 7 and 14 days of age and most preferably at 7 days of age or 14 days of age or at both 7 and 14 days of age.

The booster doses are typically administered orally in the drinking water although the booster dose can be administered by any route including by spray 10 administration. Administration of the vaccine in the drinking water can be performed by any of a number of methods known in the art. By way of example only, administration in the drinking water can be performed using the following method. First all disinfectants, sanitizers and antimicrobials are removed from the drinking water being given to the birds 24 hours prior to vaccine administration. Such water 15 free of disinfectants, sanitizers and antimicrobials is again given 24 hours after vaccination. The vaccine can then be mixed in the clean water that contains no sanitizing agents or antimicrobials. Fifteen liters of vaccine-containing water can be used for 500 birds such as chickens and ample space should be provided for all birds to drink easily. Water containing vaccine should be consumed in 2 hours or less. To 20 assure that all birds drink, water should be withheld for one to two hours prior to administration in the drinking water.

Because the dosage amount for spray administration of the vaccine or immunogenic composition is approximately the same as the oral dose in the drinking water, both dosage amounts are, preferably, about the same. Thus, for example, if  $10^6$  25 colony forming units are administered per bird by spray administration, then, preferably, about  $10^6$  colony forming units are administered per bird in the drinking water. Preferably, the initial spray administration dose and any subsequent booster dose will differ by less than 100 fold, more preferably by less than 10 fold, even more preferable by less than three fold and still more preferably by less than 10%.

30 It is preferred that the poultry to be vaccinated be of an age at which it is susceptible to the beneficial effects of the protein that is delivered by the carrier bacterium. While this may vary with species, it has been found that, such beneficial

effects are obtained in poultry that is of an age of from hatching to about 104 weeks of age. It is preferred that poultry be day-of-hatch to 52 weeks of age, more preferably from day-of-hatch to 3 weeks of age, even more preferably day-of-hatch to 2 weeks of age, still more preferably day-of-hatch to 1 week of age and most preferably that it be day-of-hatch. As used herein, the phrase "day-of-hatch" may be used interchangeably with the term "less than one day of age".

One advantage of the present method is that it is amenable to application under conditions that normally occur in commercial poultry raising operations. Typically, large commercial chicken or turkey raising operations are characterized by 10 large poultry houses having more or less automated feed and watering systems and housing over 1,000 birds per house; often over 5,000 birds per house and even over 20,000 birds per house.

The present method can be used at the hatchery or at the poultry farm on newly hatched chicks by spraying the chicks in the chick boxes, or other trays or 15 boxes, prior to their release into the brooder house or poultry house. Alternatively, either young or older poultry can be sprayed after release into the house. (See, e.g., Grieve, *Poultry Times*, p.18, September 22,1997; and Giambrone, 1997, *supra*.)

Because of the ease of application of the present method, the cost of poultry vaccination can be very low. The high cost of individual chick handling is avoided by 20 the ability to deliver a protein to dozens of chicks at one time and in a matter of seconds. Moreover, the accurate administration of the dosage of the carrier bacteria to each chick minimizes overdosing and inefficient application of the carrier bacteria.

The following examples describe preferred embodiments of the invention. Other embodiments within the scope of the claims herein will be apparent to one 25 skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

#### EXAMPLE 1

This example illustrates that spray vaccination of young chicks with a live *S. typhimurium* vaccine was as effective as direct oral administration of the vaccine in

inducing serum immunity and in producing colonization of the intestinal tract and visceral tissues by the vaccine microbe and in inducing serum immunity.

To determine the efficiency of colonization and induction of immunity by a live avirulent  $\Delta cya \Delta crp$  mutant *S. typhimurium* vaccine the present study investigated 5 the use of a coarse spray as a means to deliver the primary and booster vaccinations to day-old chicks. Duplicate groups of birds were given the vaccine by the oral route. In addition, two smaller groups of birds were given the wild-type *S. typhimurium*, UK-1 MGN-054s, by the spray and oral inoculation methods and LD<sub>50</sub>'s were determined. Colonization of the spleen, bursa, intestinal tract and cecum by the 10 vaccine strain at 7 and 20 days of age was determined for the groups of birds receiving the vaccine strain by the coarse spray route and the direct oral method of delivery. Serum antibody responses were measured by ELISA for sera recovered from 20-day-old vaccines.

**Objective:**

15 The goals of the study were: 1) to determine if young chicks vaccinated by using a coarse spray method of vaccination are as efficiently colonized by the vaccine strain compared to chicks vaccinated by the direct oral route of vaccination, and 2) to evaluate serum immunity elicited by escalating doses of the vaccine given by either coarse spray or oral administration. Since a lethal endpoint can be determined in day- 20 old chicks (but not in 3-day-old or older chicks), the wild-type *S. typhimurium* parent strain was included to determine if both methods of delivery could efficiently and comparably cause disease.

**Materials and Methods:**

25 *S. typhimurium* UK-1  $\Delta cya-12 \Delta crp-11 \chi 3985$  is an attenuated vaccine strain (see Curtiss III, et al., in *Colonization Control of Human Bacterial Enteropathogens in Poultry*, Blankenship et al., eds. Academic Press, New York, 1991, pp. 169-198). This strain was grown as a fresh late log-phase Luria broth culture and subsequently diluted in buffered saline with gelatin to the desired dose density. The vaccine was prepared to deliver escalating doses of about 10<sup>5</sup>, 10<sup>7</sup> and 10<sup>9</sup> CFU (colony forming units) to groups of 4 one-day-old white leghorn chicks. The chicks were treated with either coarse spray (droplets of approximately 140 micron diameter) using a spray 30 vaccination device (Preval Power Unit, Precision Valve Corporation, Yonkers, NY,

10703) or by direct oral vaccination administered with an 18 gauge x 7.5 mm feeding needle with a 3 mm ball attached at the end. Birds were maintained on Purina Start and Grow without coccidiostats or antibiotics.

- Concurrently, escalating doses of the wild-type *S. typhimurium* parent strain 5 MGN-054s were prepared in buffered saline with gelatin from a fresh late log-phase Luria broth culture and administered to groups of 3 day-of-hatch white leghorn chicks by either coarse spray or by direct oral delivery.

All groups of birds that received the vaccine strain on day-of-hatch were 10 administered a booster inoculation on day 14 by the same route and dosage as used per individual group for the primary inoculation. At 7 and 20 days of age, groups of birds were euthanized with CO<sub>2</sub> asphyxiation and necropsies performed to aseptically recover samples of the spleen and bursa, fecal matter and cecal contents for enumeration of the vaccine strain.

Serum was separated from blood collected during the 20-day necropsy and 15 diluted 1:200 for ELISA. Briefly, purified LPS from *S. typhimurium* was used as the test antigen. Goat anti-chicken IgA, IgM and IgG affinity-purified polyclonal antibodies EC2-001, EC2-005 and EC2-0011, respectively, (from Immunovision, Springdale, AR) were used at a dilution of 1/5000 to detect chicken IgM, IgA or IgG bound to purified LPS antigen on ELISA plates (Dynatech Laboratories Inc., 20 Alexandra, VA). A rabbit anti-goat IgG (whole molecule) alkaline phosphatase conjugate (Sigma, St. Louis, MO) was used at a dilution of 1/3000 with P-nitrophenyl phosphate (Sigma, St. Louis, MO) (1 mg/ml) in diethanolamine buffer pH 9.8 as the substrate. The reaction was stopped after 30-min. incubation with 50 µl of 3M sodium hydroxide. Absorbancies were read at 405 nm using an automated microplate 25 reader (Bio-Tek, Winooski, VT). The ELISA results were expressed as mean absorbance (405 nm) of four chickens within a treatment group. The cut-off point was taken as 2X the mean of the absorbance of sera from noninfected chickens which served as the baseline for the detection of chicken immunoglobulin isotypes by ELISA.

30 **Results and Conclusion:**

No mortality was observed in groups of birds that received doses of the vaccine up to 10<sup>9</sup> CFU administered by on days 1 and 14. The LD<sub>50</sub>'s observed in the

groups of day-old chicks that received escalating doses of the wild-type virulent parent strain by the coarse spray or oral method of delivery were approximately  $10^3$  CFU, which were the same as previously determined by oral inoculation in day-old chicks.

5       Figure 1 (a, b and c) shows recovery of the vaccine strain from the spleen and bursal tissues and feces and cecal contents of birds 7 days after receiving the vaccine strain on day-of-hatch. Figure 2 (a, b and c) shows recovery of the vaccine strain from the spleen and bursal tissues and feces and cecal contents of birds 20 days after receiving the vaccine strain on days 1 and 14. The vaccine strain colonized visceral  
10 and lymphatic tissues as well as the intestinal tract efficiently in 100% of the birds sampled. In another study comparing these two methods of vaccine delivery, similar results were observed with broiler chicks maintained on Purina Start and Gro supplemented with the recommended levels of bacitracin (BMD-50) and a coccidiostat (Coban-100). For all dose levels compared, the data generally showed no  
15 significant differences between the use of coarse spray or direct oral inoculation to deliver the *S. typhimurium*  $\chi$ 3985 vaccine strain to leghorn or broiler birds.

The levels of *Salmonella*-specific IgM, IgA and IgG detected by ELISA against *S. typhimurium* LPS in chickens administered the vaccine  $\chi$ 3985 are presented in Figure 3. Doses ranging from  $10^5$  to  $10^9$  CFU induced significant humoral antibodies using either delivery method tested. The highest dose of  $10^9$  CFU induced the greatest antibody response compared to the lower doses. IgM was the predominant immunoglobulin at 20 days of age in all birds tested in all dose groups. It has been reported that IgM is the predominant isotope 3 weeks post-vaccination by the oral routes. (Hassan and Curtiss, *Res. Microbiol.*, 141:839 - 850, 1990). Increased  
20 antibody responses, which are reflected by higher OD<sub>405</sub> measurements, were observed in the present study in birds that received  $10^7$  or  $10^9$  CFU by spray compared to birds that received  $10^7$  or  $10^9$  CFU by direct oral method of delivery.  
25

#### EXAMPLE 2

30       This example shows the efficacy of spray vaccination of a live avirulent *S. typhimurium* vaccine in preventing colonization of the GI tract and intestinal organs by wild-type *S. typhimurium*.

Fifty-five one-day-old SPF white leghorn chicks were wing banded for use in this trial. At day-of-age, 30 birds were vaccinated by coarse spray with  $1 \times 10^7$  CFU per dose of *S. typhimurium* vaccine carried in approximately 0.3 ml per bird using a Preval Power Unit spray device, from Precision Valve Corp., Yonkers, NY 10703.

- 5     The vaccine was χ3985, U.S.D.A. Product Code 19C1.01 (Maine Biological Laboratories, Inc., Waterville, ME). Twenty birds received 0.3 ml per bird of distilled water by coarse spray and served as controls. Five additional birds were held as contemporary controls. Vaccinates and controls were housed separately in Horsfall isolation units. At two weeks of age, the vaccinates were boosted in the drinking
- 10    water with  $1 \times 10^7$  CFU per dose of the same vaccine. At six weeks of age all birds in both treatment groups were individually challenged with an oral dose of  $1 \times 10^6$  CFU in 0.1 ml of wild type *S. typhimurium*.

Cloacal swabs were collected from each bird of both treatment groups prior to challenge and at the time of sacrifice to monitor for *Salmonella sp*. Swabs were

15    placed in 5 ml of TBGH broth and incubated for 24 - 36 hours at 42°C. After about 36 hours, the broth was streaked onto brilliant green agar + 35 µg/ml novobiocin (BGAN) and incubated at 42°C.

At seven weeks of age all birds were euthanized and tissues were recovered and cultured for the wild type *S. typhimurium* as follows: Approximately 5 gm each

20    of the spleen, liver, kidney and any organ displaying visible lesions were aseptically removed from each bird. Spleen, liver and kidney tissues obtained from the same bird were pooled. Any organ displaying visible lesions was collected and processed separately. In addition, a 10 mm sample of the duodenum, ilea and large intestine tissues with contents were aseptically obtained from each bird and processed

25    individually. In addition, 1 gram of the ceca with contents were collected from each bird and processed similarly.

To culture for *Salmonella sp*. tissues were placed in a sterile Whirl®Pak bag, macerated in a Stomacher blender, and 5 ml of tetrathionate brilliant green Hajna (TBGH) broth added to each bag. The tissue bags were incubated for 24 - 36 hrs at

30    42°C, following which a loopful from each culture was streaked onto BGAN. Plates were examined after 36 hours of incubation at 42°C for characteristic CFU on BGAN agar. After the bag had been incubated for about 48 hours, 1 ml of the TBGH from

the bag culture was transferred to a tube of 4 ml of fresh TBGH broth, the tube incubated for 5 days, and then streaked onto BGAN agar. Plates were examined after 36 hours of incubation at 42°C. An agglutination test with group B *Salmonella* antiserum was performed on at least one colony per plate from all plates to confirm 5 the presence of the wild type challenge strain *S. typhimurium* F98.

Table 1. Experimental design for vaccination with avirulent *S. typhimurium* and challenge with wild-type *S. typhimurium* in chickens.

GROUP	TREATMENT*	AGE AT CHALLENGE	CHALLENGE CULTURE	NUMBER OF BIRDS
1	Vaccine	6 weeks	wild-type <i>S. typhimurium</i>	30
2	Distilled water	6 weeks	wild-type <i>S. typhimurium</i>	20
3	none	N/A	none	5

10

\*Either vaccine or distilled water was administered on day 1 by coarse spray and again on day 14 in the drinking water.

#### Results:

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No clinical reaction to the vaccine was observed after any vaccination. The vaccine strain was isolated from one of thirty birds 8 days after vaccination, but was not recovered from any of the birds thereafter up to the day of challenge. No deaths occurred during the course of the trial.

20

Culture results for all pre- and post-challenge cloacal swabs are presented in Table 2 below. The vaccine organism was isolated on day 8 post-vaccination from one of the 30 birds; all vaccinated birds were culture negative when sampled again on day 42 post-primary vaccination. Seven days after challenge, wild-type *S. typhimurium* were cultured from the cloacal swabs of 13% of the vaccinated birds compared to 40% of the non-vaccinated birds receiving distilled water only.

Table 2. Number of culture positive cloacal swabs sampled from treated and non treated birds pre- and post-challenge with wild-type *S. typhimurium*.

GROUP	Treatment	PRE-CHALLENGE <sup>1</sup>		POST-CHALLENGE <sup>2</sup>
		Day 8	Day 42	Day 49
1	<i>S. typhimurium</i> vaccine	1/30 (3%)	0/30 (0%)	4/30 (13%) <sup>3</sup>
2	Non-treated, challenged controls	0/20 (0%)	0/20 (0%)	8/20 (40%)
3	Non-treated, non challenged controls	0/5 (0%)	0/5 (0%)	0/5 (0%)

5      <sup>1</sup>Number of chickens showing positive culture for *S. typhimurium* vaccine strain/total number of chickens.

10     <sup>2</sup>Number of chickens showing positive cultures for wild-type challenge *S. typhimurium*/total number of chickens.

15     <sup>3</sup>Significantly different from non-treated, challenged controls using Chi-square test (P<0.05).

The vaccinated and non-vaccinated birds also differed in the degree of colonization of the GI tract and the spleen, liver and kidney alternatively (see Table 3). Wild-type *S. typhimurium* were cultured from pooled organ tissues of 85% of the non-vaccinated control birds compared to 13% of the vaccinated birds ( $P < 0.05$ ). A significant reduction in the number of culture positive ileal and large intestine samples was found from vaccinated and non-vaccinated birds ( $P < 0.05$ ). A significant reduction was also found in the number of culture positive cecal samples from vaccinated and non-vaccinated birds ( $P < 0.05$ ). No differences could be determined between the numbers of culture positive duodenal samples from the vaccinated and non-vaccinated birds.

Table 3. Culture of *S. typhimurium* in broiler age birds vaccinated with avirulent live *S. typhimurium* challenged with wild-type *S. typhimurium*.

GROUP	VACCINATION (Day:CFU)	CHALLENGE at 6 weeks (CFU)	CULTURE RESULTS (No. positive/tested (%))				
			Organ Pool	Duodenum	Ilea	Large Intestine	Ceca
1	Day 1: $3.6 \times 10^6$	$1 \times 10^6$	6/30		8/30	5/30	7/30
	Day 14: $6.8 \times 10^6$		(20%) <sup>1</sup>	1/30 (3%)	(27%) <sup>1</sup>	(17%) <sup>1</sup>	(23%) <sup>1</sup>
2	None	$1 \times 10^6$	17/20		12/20	13/20	10/20
3	None	None	0/5	0/5	0/5	0/5	0/5

- 5   <sup>1</sup> Significantly different from group receiving no vaccination and challenged with wild-type microbe using Chi-square test ( $P < 0.05$ ).

The modified live *S. typhimurium* vaccine provided protection to vaccinated birds against artificial challenge with invasive wild-type *S. typhimurium*. This challenge trial showed that birds vaccinated at 1 and 14 days of age with the live *S. typhimurium* vaccine had a statistically significant advantage over control birds. There was a significant reduction in the number of broiler age birds in both treatment groups in internal organ colonization by the wild-type *S. typhimurium* ( $P < 0.05$ ). Protection at the gut level was apparent as fewer numbers of vaccines were culture positive from ileal, large intestine and cecal samples tested than found from these tissues from non-vaccinated birds ( $P < 0.05$ ). The duodenum does not appear to be a target tissue for *S. typhimurium* in that only 10% of the non-vaccinated control birds were colonized by the wild-type. In addition, a significant reduction in the number of birds showing positive cloacal culture swabs for the wild-type challenge organism was noted in the vaccine-treated birds as compared to the non-treated birds.

The data from this study demonstrate that *S. typhimurium* vaccine administered to chicks on day one as a coarse spray and in drinking water at day 14 significantly reduces colonization of the GI tract and invasion and colonization of visceral organs by wild-type *S. typhimurium* in broiler age birds.

25

### EXAMPLE 3

This example illustrates the efficacy of spray administration of a live avirulent *S. typhimurium* vaccine in preventing colonization of internal organs following oral challenge with either wild-type *S. enteritidis* or *S. heidelberg*.

Table 4 shows the experimental design of the spray vaccination studies in which chickens were challenged with either *S. heidelberg* or *S. enteritidis*.

5 Table 4. Experimental design for vaccination with *S. typhimurium* vaccine and challenge with *S. heidelberg* or *S. enteritidis* in chickens.

GROUP	VACCINE	AGE AT VACCINATION	CHALLENGE	AGE AT CHALLENGE	NO. OF BIRDS
1	Chi3985, Product Code 19C1.01	Days 1 & 14	<i>S. heidelberg</i>	6 weeks	33
2	None	None	<i>S. heidelberg</i>	6 weeks	20
3	None	None	None	None	10
4	Chi3985, Product Code 19C1.01	Days 1 & 14	<i>S. enteritidis</i>	6 weeks	33
5	None	None	<i>S. enteritidis</i>	6 weeks	20
6	None	None	None	None	10

10 *S. heidelberg* Study

Sixty-three one-day-old SPF white leghorn chicks (SPAFAS Inc., Storrs, CT) were wing banded for use in this trial. At one day of age, 33 birds (Group 1) were vaccinated by coarse spray with  $1 \times 10^7$  CFU per dose of vaccine Chi3985, U.S.D.A. Product Code 19C1.01 delivered in approximately 0.35 ml volume per bird using a 15 Preval Power Unit (Precision Valve Corp., Yonkers, NY) spray device. Twenty birds (Group 2) received 0.3 ml per bird of distilled water by coarse spray and served as controls, ten additional birds (Group 3) were held as non-vaccinated, non-challenged controls. Vaccinates and controls were housed separately in Horsfall isolation units.

At two weeks of age, the vaccinees were boosted in the drinking water with 20  $9.6 \times 10^6$  CFU per dose of the same vaccine; for consumption by each bird in 5.1 ml of water. Serum samples were collected prior to challenge from six-week-old birds in Groups 1 and 2 to assess *Salmonella* antibodies by ELISA. Cloacal swabs were also taken from each bird at this time to culture for the presence of *Salmonella* sp. At six weeks of age, all birds in Groups 1 and 2 were individually challenged with an oral

dose of  $1.6 \times 10^8$  CFU in 1.0 ml of wild-type nalidixic acid-resistant *S. heidelberg*. Four days later, all birds were swabbed, euthanized and tissues were recovered and cultured for the wild-type *S. heidelberg* strain as follows. Approximately 10 gm each of the spleen, liver, kidney and any organ displaying visible lesions were aseptically removed from each bird. Spleen, liver and kidney tissues obtained from the same bird were pooled. Any organ displaying visible lesions was collected and processed separately. In addition, a 10 mm sample of the duodenum, ilea and large intestine were aseptically obtained from each bird and processed similarly. In addition, 10 mm sample of the ceca with contents expressed were collected from each bird and processed similarly.

To culture for *Salmonella* sp., tissues were placed in a sterile Whirl®Pak bag, 25 ml of tetrathionate brilliant green Hajna (TBGH) broth added to each bag and the sample was macerated in a Stomacher blender. The tissue bags were incubated for 40 hrs at 42°C, following which a 10 µl loopful from each culture was streaked onto brilliant green agar + 35 µg/ml novobiocin (BGAN) and xylose-lysine-tergitol 4 agar + 100 µg/ml nalidixic acid (XLT4-Nal). Plates were examined after 24 hours of incubation at 42°C for characteristic colonies on XLT4-Nal and BGAN agar. The enrichment broth cultures were incubated for an additional 24 hours. The cultures corresponding to negative plates were re-streaked onto XLT4-Nal and BGAN, incubated at 42°C for 24 hours and observed for characteristic colonies. An agglutination test with *Salmonella* O group specific (Group B) antiserum was performed on at least one colony per plate from all plates to confirm the presence of the wild-type challenge strain *S. heidelberg*.

Cloacal swabs were collected from each bird of all treatment groups prior to challenge and at the time of sacrifice to monitor for *Salmonella* sp. Swabs were placed in 5 ml of TBGH broth and incubated for 24 - 40 hours at 42°C. After about 40 hours, the broth was streaked onto BGAN and incubated at 42°C. An agglutination test with *Salmonella* O group specific (Group B) antiserum was performed on at least one colony per plate from all plates to confirm the presence of the wild-type challenge strain of *S. heidelberg*.

**Results:**

- No clinical reaction to the vaccine was observed after any vaccinations. No deaths attributed to the vaccine occurred during the course of the trial. One bird in the vaccinated group died from injury on day 49 of the trial. There were no birds in
- 5 Group 1 that showed any vaccine positive cloacal cultures when sampled on day 42 post-vaccination.

Culture results for all pre- and post-challenge cloacal swabs are presented in Table 5 below. The vaccine organism was not recovered from any of the birds in Group 1 before challenge. Four days after receiving the wild-type challenge

10 organism, 50% of the vaccinated birds showed positive cloacal cultures of the wild-type organism while 70% of the non-vaccinated control birds were culture positive. These data show no statistical difference between the two groups in showing positive cloacal cultures for the wild-type organism.

15 Table 5. Number of culture positive cloacal-swab samples from treated and untreated birds pre- and post-challenge with wild-type *S. heidelberg*.

TREATMENT	PRE-CHALLENGE <sup>1</sup> (Day 42)	POST-CHALLENGE <sup>2</sup> (Day 46)
Group 1: <i>S. typhimurium</i> vaccine	0/33 (0%)	16/32 (50%) <sup>3</sup>
Group 2: Non-treated, challenged controls	0/20 (0%)	14/20 (70%) <sup>3</sup>
Group 3: Non-treated, non-challenged controls	0/10 (0%)	0/10 (0%)

20 <sup>1</sup> Number of chickens showing positive culture for *S. typhimurium* vaccine strain/total number of chickens.

<sup>2</sup> Number of chickens showing positive cultures for wild-type challenge *S. heidelberg*/ total number of chickens.

25 <sup>3</sup> Significantly different from non-vaccinated non-challenged group using Chi-square test ( $P < 0.05$ ).

Groups of vaccinated and non-treated birds were challenged at 6 weeks of age with an oral dose of live wild-type *S. heidelberg* to assess protection against internal organ tissue invasion and digestive tract colonization (See Table 6). A significant difference was found between the vaccinated and non-vaccinated groups challenged with wild-type *S. heidelberg*; twenty-five percent of the vaccinated birds challenged with wild-type were culture positive in pooled organ tissues compared to 70% of the non-vaccinated control birds ( $P < 0.05$ ). A significant reduction in the number of culture positive digestive tract pooled samples was found from vaccinated compared to non-vaccinated birds ( $P < 0.05$ ). No differences in the number of culture positive cecal samples were seen between the vaccinated and non-vaccinated birds. Non-treated, non-challenged control birds were free of *Salmonella* sp.

Table 6. Protection of broiler age birds vaccinated with live avirulent *S. typhimurium* and challenged with wild-type *S. heidelberg*

GROUP	TREATMENT	ORGAN POOL	DIGESTIVE TRACT POOL	CECA
1	Vaccine + challenge	8/32 (25%) <sup>1</sup>	10/32 (31%) <sup>1</sup>	21/32 (66%)
2	Non-vaccinated challenged controls	14/20 (70%)	17/20 (85%)	17/20 (85%)
3	Non-vaccinated, non-challenged controls	0/10 (0%)	0/10 (0%)	0/10 (0%)

<sup>1</sup> Significantly different from non-vaccinated, challenged group using Chi-square test ( $P < 0.05$ ).

#### 20 *S. enteritidis* Study:

Table 4 shows the experimental design of the *S. enteritidis* efficacy study. Sixty-three one-day-old SPF white leghorn chicks (SPAFAS Inc., Storrs, CT) were wing banded for use in this trial. At day-of-age, 33 birds (Group 4) were vaccinated by coarse spray with  $1 \times 10^7$  CFU per dose of  $\chi$ 3985, U.S.D.A. Product Code 19C1.01 vaccine carried in approximately 0.35 ml volume per bird using a Preval Power Unit

spray device (Precision Valve Corp., Yonkers, NY). Twenty birds (Group 5) received 0.3 ml per bird of distilled water by coarse spray and served as controls, ten additional birds (Group 6) were held as non-vaccinated, non-challenged controls. Vaccinates and controls were housed separately in Horsfall isolation units.

5 At two weeks of age, the vaccinees were boosted in the drinking water with  $9.6 \times 10^6$  CFU per dose of the same vaccine for consumption by each bird in 5.1 ml of water. Serum samples were collected prior to challenge from 6-week-old birds in Groups 4, 5 and 6 to assess *Salmonella* antibodies by ELISA. Cloacal swabs were also taken from each bird at this time to culture for the presence of *Salmonella* sp. At 10 six weeks of age, all birds in Groups 4 and 5 were individually challenged with an oral dose of  $4.5 \times 10^7$  CFU in 1.0 ml of wild-type nalidixic acid-resistant *S. enteritidis*. Seven days later, all birds were swabbed, euthanized and cultured for the wild-type *S. enteritidis* strain by the method described above in the *S. heidelberg* efficacy study, except that the agglutination tests were run with *Salmonella* O group 15 specific (Group D) antiserum rather than Group B antiserum.

### Results

No clinical reaction to the vaccine was observed after any vaccinations. No deaths attributed to the vaccine occurred during the course of the trial. One bird in the 20 vaccine-treated group died, presumably from injury received after heart puncture in an effort to collect blood for serum antibody evaluation.

Culture results for all pre- and post-challenge cloacal swabs are presented in Table 7 below. The vaccine organism was not recovered from any of the birds in Group 4 before challenge. Seven days after receiving the wild-type challenge 25 organism, 41% of the vaccinated birds showed positive cloacal cultures for the wild-type organism while 63% of the non-vaccinated control birds were culture positive. These data show no statistical difference between the two groups in positive cloacal cultures for the wild-type organism.

Table 7. Number of culture positive cloacal swabs samples from vaccinated and non-vaccinated birds pre- and post-challenge with wild-type *S. enteritidis*.

GROUP	TREATMENT	PRE-CHALLENGE <sup>1</sup> (Day 42)	POST-CHALLENGE <sup>2</sup> (Day 49)
4	Vaccine + challenge	0/33 (0%)	13/32 (41%) <sup>3</sup>
5	Non-vaccinated, challenged controls	0/20 (0%)	12/19 (63%) <sup>3</sup>
6	Non-vaccinated, non-challenged controls	0/10 (0%)	0/10 (0%)

<sup>1</sup> Number of chickens showing positive culture for *S. typhimurium* vaccine strain/total number of chickens.

<sup>2</sup> Number of chickens showing positive cultures for wild-type challenge *S. typhimurium*/total number of chickens.

<sup>3</sup> Significantly different from non-vaccinated, non-challenged controls using Chi-square test ( $P < 0.05$ ).

The vaccinated and non-vaccinated birds also differed in the degree of colonization of the GI tract and the spleen, liver and kidney collectively as shown in Table 8. A significant difference was found between the vaccinated and non-vaccinated groups challenged with wild-type *S. enteritidis*; nine percent of the vaccinated birds challenged with wild-type were culture positive in pooled internal organ tissues compared to 58% of the non-vaccinated control birds ( $P < 0.05$ ). A significant reduction in the number of culture positive digestive tract pooled samples was found from vaccinated and non-vaccinated birds ( $P < 0.05$ ). No differences in the number of culture positive cecal samples were seen between the vaccinated and non-vaccinated birds. Non-treated, non-challenged control birds were free of *Salmonella* sp.

Table 8. Protection of broiler age birds vaccinated with a modified live *S. typhimurium* vaccine after oral challenge with wild-type *S. enteritidis*.

GROUP	TREATMENT	ORGAN POOL	DIGESTIVE TRACT POOL	CECA
4	Vaccine + challenge	3/32 (9%) <sup>1</sup>	4/32 (13%) <sup>1</sup>	16/32 (50%)
5	Non-vaccinated, challenged controls	11/19 (58%)	7/19 (37%)	11/19 (58%)
6	Non-vaccinated, Non-challenged controls	0/10 (0%)	0/10 (0%)	0/10 (0%)

5      <sup>1</sup> Significantly different from non-vaccinated challenged controls using Chi-square test ( $P < 0.05$ ).

10     Serum samples were screened for IgG antibody to *S. typhimurium* lipopolysaccharide (LPS) by ELISA. Samples were diluted 1:100 and added to duplicate wells coated with commercially prepared *S. typhimurium* LPS. HRP-conjugated rabbit anti-chicken IgG at 1:30,000 was used for detection. Of the birds vaccinated with *S. typhimurium* vaccine, 52% developed a strong response to the vaccine with OD<sub>490</sub> measurements greater than 0.3; nine percent responded positively in the mid-range of 0.2 - 0.3 and 39% measured in the low-range of 0.05 - 0.1. Sera from control birds averaged OD<sub>490</sub> measurements of 0.005 ± 0.009, well below all measurements derived from sera from vaccinated birds.

### Conclusion:

20     Day-of-hatch, spray vaccination with the live avirulent *S. typhimurium* vaccine, χ3985, U.S.D.A. Product Code 19C1.01, followed by oral administration of the vaccine in the drinking water at day 14 did not produce any adverse reaction in chickens. The vaccine strain was not recovered from cloacal swabs of spray vaccinated birds when sampled at 6 weeks of age or just prior to challenge with the wild-type *S. enteritidis* or *S. heidelberg* organisms. All vaccinated birds that were

tested mounted a serologic response when exposed to the *S. typhimurium* vaccine as compared to non-vaccinated birds.

The data generated from this trial demonstrate that the *S. typhimurium* vaccine provided significant protection to reduce the level of colonization of internal organs by either *Salmonella heidelberg* or *S. enteritidis*. Although no differences were seen in the level of colonization by the wild-type challenge strains in the ceca of vaccinated compared to non-vaccinated birds, significant protection from *S. heidelberg* or *S. enteritidis* colonization of the digestive tract was conferred by vaccination.

10

#### EXAMPLE 4

This shows the efficacy of spray vaccination using a live avirulent *S. typhimurium* vaccine in protecting broiler age birds against internal organ colonization after challenge with wild-type *S. enteritidis*.

Fifty-five one-day-old SPF white leghorn chicks (HyVac, Adel, IA) were wing banded for use in this trial. At day-of-age, 30 birds (Group 1) were vaccinated by coarse spray with approximately  $1 \times 10^7$  CFU per dose of  $\chi$ 3985, U.S.D.A. Product Code 19C1.01 vaccine (produced by Maine Biological Laboratories, Inc., Waterville, ME), carried in approximately 0.3 ml volume per bird using a Preval Power Unit spray device (Precision Valve Corp., Yonkers, NY). Twenty birds (Group 2) received 0.3 ml per bird of distilled water by coarse spray and served as controls, five additional birds (Group 3) were held as contemporary controls. Vaccinates and controls were housed separately in Horsfal isolation units.

25

Table 9. Experimental design for vaccination with *S. typhimurium* vaccine and challenged with *Salmonella enteritidis*.

GROUP	VACCINE	AGE AT VACCINATION	CHALLENGE	AGE AT CHALLENGE	NO. OF BIRDS
1	Chi3985, Product Code 19C1.01	Days 1 & 14	<i>S. enteritidis</i>	6 weeks	30
2	None	None	<i>S. enteritidis</i>	6 weeks	20
3	None	None	None	N/A	5

- At two weeks of age, the vaccines were boosted in the drinking water with approximately  $1 \times 10^7$  CFU per dose of the same vaccine (one dose in 15 ml of water per bird). At six weeks of age all birds in the vaccine-treated and control groups were individually challenged with an oral dose of  $4 \times 10^7$  CFU in 0.5 ml of wild-type *S. enteritidis*.
- 5    *enteritidis*. Cloacal swabs were also collected 5 days post challenge from each bird to assess the number of wild-type cloacal swabs. All birds were euthanized 5 days post challenge, necropsied and tissue samples cultured for the wild type *S. enteritidis* as described in Example 2.

10    **Results:**

No clinical reaction to the vaccine was observed during the vaccination period. No deaths occurred in all treatment groups during the course of the trial.

- Groups of vaccinated and non-treated birds were challenged at six weeks of age with an oral dose of live wild-type *S. enteritidis* to assess protection against
- 15 internal organ tissue invasion and GI tract colonization. (See Table 10). A significant difference was found between the vaccinated and non-vaccinated groups challenged with wild-type *S. enteritidis*; ninety-five percent of the non-vaccinated control birds challenged with wild-type were culture positive in pooled organ tissues after challenge compared to 20% of the vaccinated birds ( $P < 0.01$ ). A significant reduction in the number of culture positive duodenal samples was found within the vaccine-treated group and control group ( $P < 0.01$ ). No differences were found between the number of *S. enteritidis* culture positive ileal, large intestine or cecal samples from vaccinated and non-vaccinated birds.
- 20

Table 10. Protection of broiler age birds vaccinated with a modified live *S. typhimurium* vaccine after oral challenge with wild-type *S. enteritidis*.

GROUP	TREATMENT	ORGAN POOL	DUODENUM	ILEA	LARGE INTESTINE	DIGESTIVE TRACT (Pooled data)	CECA
1	Vaccine	6/30 (20%) <sup>1</sup>	2/30 (7%) <sup>1</sup>	20/30 (67%)	26/30 (87%)	27/30 (90%)	29/30 (97%)
2	Controls	21/22 (95%)	12/22 (55%)	15/22 (68%)	22/22 (100%)	22/22 (100%)	22/22 (100%)
3	Isolated controls	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)

<sup>1</sup> Significantly different from non-vaccinated, challenged control groups using Chi

5 Square Test ( $\alpha = 0.01$ ).

Eighty-six percent of the vaccine treated birds showed positive cloacal cultures for the wild-type challenge organism compared to 100% of the birds in the non-vaccinated control group.

10 **Conclusion:**

Day of hatch spray vaccination with the live  $\chi 3985$ , U.S.D.A. Product Code 19C1.01 vaccine, followed by oral administration of the vaccine in the drinking water at day 14 did not produce any adverse reaction in chickens.

15 Although protection from wild-type colonization was not apparent in the digestive tract and cecal samples of vaccinated birds, significant protection against internal organ colonization by the wild-type *S. enteritidis* was demonstrated in the internal organs of vaccine-treated, challenged birds compared to non-vaccinated, challenged birds.

20

### EXAMPLE 5

This example illustrates that spray vaccination of chicks with modified live *S. typhimurium* vaccine was safe and efficacious in reducing *Salmonella* contamination under field conditions of normal large-scale commercial poultry production.

**Purpose:**

25 The purpose of this trial was to evaluate the safety and potential of the  $\chi 3985$ , U.S.D.A. Product Code 19C1.01 vaccine to reduce *Salmonella* contamination under normal large scale commercial poultry production conditions. The safety of the vaccine was monitored by measuring the ability of the product to spread and the survival of the chickens during the grow-out period. The efficacy of the product was  
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monitored by bacteriological analyses of post-chill carcass rinses after processing, average weight and the percent condemnations at processing.

#### **Materials and Methods:**

Three geographically distinct farms with paired houses were chosen such that 5 each poultry house accommodated a minimum of 16,000 birds. This allowed a triple-replicate trial utilizing more than 115,000 birds consisting of control birds and birds treated with the modified live *S. typhimurium* vaccine. An effort was made to identify trial poultry farms that had a known background history of persistent *Salmonella* contamination. Table 11 identifies the sites and study parameters of the three trials.

10

Table 11. Description of vaccine field safety trials

FIELD TRIAL SITE	VACCINE SERIAL USED	NO. OF CHICKENS		TIME OF VACCINATION (Days after hatching)		DURATION OF TRIAL (DAYS)
		VACCINATED	CONTROL	FIRST	BOOSTER	
1	7002	21,000	21,000	1	14	48
2	7003	20,500	20,500	1	17	42
3	7004	16,000	16,000	1	15	64

#### **Production of Prelicensing Serials:**

15 The modified live χ3985, U.S.D.A. Product Code 19C1.01 vaccine prelicensing serials were manufactured following the Outline of Production filed and approved March 20, 1995.

#### **Trial Procedure:**

20 At all farm sites, the normal commercial vaccination, feeding and watering regimens of that particular poultry establishment were followed. Hatchlings received Marek's vaccine either in ovo or at day of hatch and Newcastle/bronchitis vaccine by spray at day of hatch. A booster bronchitis vaccination was administered in both houses on each farm site on day 16 - 18 of the study.

25 **Application Procedures for the *S. typhimurium* Vaccine:**

1. **Spray application at day of age:**

A portable spray box apparatus (supplied and operated by Merial Select, Atlanta, GA) was set up in the hatchery so that incoming boxes of birds were vaccinated prior to setting the chicks out on the floor of the house. Vaccine was

supplied as a lyophilized formulation in a glass vial. The vaccine was mixed into clean, non-chlorinated water according to the package instructions. The spray equipment was pre-calibrated manually to deliver approximately 700 ml to 1 liter over 10,000 chicks under a pressure of 40 - 50 psi using 4 coarse anvil fantail spray nozzles to deliver 50 - 100 micron sized droplets. This delivered approximately 0.07 - 0.1 ml per chick.

5        2. Drinking water application at two weeks of age:

Two-week old birds were deprived of drinking water for a period of two to four hours before allowing birds in the treated house on each farm access to the 10 vaccine water. The Merial Select Bag Boost system was implemented for calibrated delivery of the vaccine. The required quantity of the *S. typhimurium* vaccine was mixed according to the package instructions to a final volume of diluent sufficient to administer one dose per bird over a two hour period through all house water lines. This vaccine water was the bird's only source of water over this two-hour period to 15 ensure that all birds had the opportunity to drink sufficient vaccine. The well water used in the houses on all farms was tested prior to water vaccination and was found to be negative for chlorine residue and within a pH range of 6.0 - 7.0.

Sampling and enrichment culture procedures during the trial:

To monitor the presence of indigenous wild-type *Salmonella spp.* in the 20 environment and in the chick, base-line samples of meconia from chick papers were collected in the hatchery, while litter drag swabs, feed and water were collected in the houses for bacteriological analysis as described below. Drag swabs of the litter, feed and water samples were collected periodically throughout the trials at each test site.

1. Chick papers and swabs:

25        A random sample of 12 - 18 chick papers and swabs representative of each breeder flock was collected from individual chick transport boxes before administering the vaccine. Each chick paper selected containing meconia was placed in individual plastic bags, which were sealed and labeled. Sterile gauze pads wetted with sterile skim milk were used to swab additional chick papers, then placed in 30 plastic Whirl® Pak bags, sealed and labeled. All samples were immediately transported on ice packs to the laboratory for analysis of *Salmonella spp.* to determine a base-line level of contamination of incoming chicks.

2. Feed and water samples:

Before the start of the study, a 40g sample of feed was taken from the internal bin auger inflow stream from each farm. These samples were placed in Whirl® Pak bags and labeled. In addition, 100 ml of water was collected at the source after the filter and 100 ml of water collected from the nipple drinker from each house. The collected water was immediately analyzed for chlorine residue and range of pH. Samples of feed and water were collected every two weeks throughout the trial period for each farm, labeled and transported on ice packs to the laboratory for bacteriological analysis.

10 3. Drag swab samples of house litter:

Before the start of each trial, drag swab samples of the litter were collected from each house on each farm to determine base-line levels of *Salmonella* contamination. Additional drag swab samples of each house on each farm were collected every two weeks throughout the trial period. Drag swab assemblies were constructed following the National Poultry Improvement Plan and Auxiliary Provisions (National Poultry Improvement Plan and Auxiliary Provisions manual, United States Department of Agriculture, Animal and Plant Health Inspection Service (APHIS 92-55-017), April 1993). The bags were sealed, labeled and transported on ice packs to the laboratory for bacteriological analyses.

20 4. Culture procedures:

Buffered peptone water (International Bioproducts Inc., Redman, WA; Difco Inc., Detroit, MI) was used to pre-enrich feed, water, chick swabs, chick papers and drag swabs and served as the rinse to assess the bacterial load on carcasses. Tetrathionate brilliant green-Hajna broth (Northeast Laboratories Inc., Waterville, ME) was used as selective media for enrichment of *Salmonella* sp. or the vaccine. Tetrathionate brilliant green agar supplemented with 35 µg novobiocin/ml (Sigma, St. Louis, MO) was used to identify characteristic growth of *Salmonella* sp. or the vaccine. Confirmation of *Salmonella* was accomplished following the procedures outlined in the FSIS Sample Collection Guidelines and Procedure for Isolation and Identification of *Salmonella* from Raw Meat and Poultry Products. All positive cultures were sent to National Veterinary Services Laboratory (Ames, IA) for serotyping.

**5. Bacteriological assessment of whole carcasses:**

Fifty carcasses were randomly selected post-chill after processing from each house. The FSIS Sample Collection Guidelines and Procedure for Isolation and Identification of *Salmonella* from Raw Meat and Poultry Products (Food Safety Inspection Service Sample Collection Guidelines and Procedure for Isolation and Identification of *Salmonella* from Raw Meat and Poultry Products, *Federal Register* Vol. 61, No. 144, 7/25/96, page 38923), was followed to collect whole carcass rinses and to assess the presence or absence of *Salmonella sp.* and/or vaccine residues. In addition, PCR (BACS System, Qaulicom, Wilmington, DE) was used to verify samples that were positive for agglutination by antisera to specific *Salmonella* O-antigen groups (Disco Inc., Detroit, MI), but where pure cultures could not be obtained.

**Statistical Methods:**

The  $\chi^2$ -Square test was used to compare parametric results between vaccine-treated and control houses.

**Results and Discussion:**

**Trial at Site No. 1:**

**1. Analyses of base-line samples:**

Analyses of base-line samples consisting of chick papers and paper swabs, water, feed and drag swabs of the house litter collected prior to the start of the trial revealed that the feed from the control house was positive for *Salmonella mbandaka* and chicks from two of three breeder flocks showed positive cloacal cultures for *Salmonella heidelberg*. Chicks from one of the breeder flocks showed positive cloacal cultures for organisms suspected to be of O-antigen group C<sub>3</sub>, however a pure isolate could not be recovered. No other *Salmonella sp.* or vaccine organisms could be recovered from feed, water or from drag swabs of the litter from either house on the farm for the remainder of the 6-week grow-out period of the trial.

**2. Livability:**

Livability data were collected weekly by the grower for each house on the farm. Table 12 shows the numbers of birds that expired during the grow-out period of the trial. No difference was seen in the loss of birds between the control and treated

house where the percent total loss for both houses was 2.8%, respectively. The average percent mortality for a period coinciding with the trial for the region of the country in which Site #1 was located was 4.4 - 4.7% (*The Poultry Informed Professional*, Department of Avian Medicine, University of Georgia, Athens, GA, 5 January, 1998).

Table 12. Mortality of birds during the grow-out period for Site #1 trial.

	CONTROL	TREATED
1st Week	205	210
2nd Week	117	87
3rd Week	57	55
4th Week	62	63
5th Week	74	81
6th Week	81	87
Total mortality headcount	596	583 <sup>1</sup>
Percent total	2.8%	2.8%

<sup>1</sup> No significant difference was found using Chi-square test (P < 0.05)

10

The historical livability percentile for Site #1 ranged from 95.5 to 99.6 in 1996 - 1997. The livability percentile of 97.2 for both the control and treated birds was within this range. Based on these data, the vaccine did not have any adverse effect on the livability of the birds.

15 3. FSIS Inspector's condemnation report at processing:

The USDA Inspector's condemnation report for each house is presented in Table 13. The Inspector's report indicated an increase of airsacculitis and inflammatory process (IP) in the carcasses from the treated house over the control house. Average values for the region were obtained from *The Poultry Informed Professional* - for a week during the trial.

Table 13. Percent condemnations at processing for Site #1 trial.

CONDEMNATION CAUSE	CONTROLS	TREATED	AVERAGE % FOR REGION
Leukosis	0.02	0.02	0.03
Septicemia/Toxemia	0.25	0.28	0.35
Tumors	0.28	0.15	N/A <sup>1</sup>
Airsacculitis	0.14	0.51	0.17
Synovitis	0	0	N/A
Inflammatory process	0.64	2.06	0.14
Overscald	0	0	N/A
Bruises	<0.01	<0.01	0.02
Tuberculosis	0	0	N/A
Cadavers	<0.01	<0.01	N/A
Contaminated	<0.01	<0.01	N/A
Dead on arrival	0.3	0.24	N/A
Total condemnations	1.33	3.02 <sup>2</sup>	

<sup>1</sup> N/A indicates data not available.

<sup>2</sup> Significantly different from control group using Chi-square test (P < 0.05)

5

The historical data provided by the collaborator shows that this test farm had experienced cyclic episodes of respiratory disease in the past two years as airsacculitis percentiles had been elevated during the winter months. The flock that was on the farm prior to this vaccinated test flock showed airsacculitis percentiles of 1.71,

10 compared to 0.51 for the birds in the treated house from this trial. There are no documented cases of *Salmonella spp.* causing airsacculitis or inflammatory process. In addition, the carcass inspection process was not blinded as the USDA Inspector in Charge required identification of the treated birds. Airsacculitis and IP percentiles were not different between controls and vaccine-treated birds in two subsequent trials

15 described in this report. Based on these reasons, an assessment cannot be made for the cause of these conditions as attributed to use of the vaccine product.

#### 4. Evaluation of the carcass weight at processing.

The average weight of the bird at processing is an indicator of performance of the bird during grow-out period. An examination of eight grow-out cycles for Site #1

20 showed average bird weights ranging from 4.36 to 4.79 lbs for birds up to 49 days of age; the treated bird weights fell within this range averaging 4.84 lbs compared to 4.85 lbs for control birds at 48 days of age. Based on these average weight data, the

vaccine did not affect the ability of the birds to maintain a level of performance expected by the producer.

##### 5. Carcass rinse evaluation.

The results of carcass rinse evaluation are shown in Table 14. The number of 5 *Salmonella* positive samples as identified by PCR and O-antigen antisera agglutination from carcass rinses was significantly less in the treatment group than that found in the control group ( $p \leq 0.05$ ). Neither the modified live *S. typhimurium* organisms nor any indigenous *Salmonella sp.* was identified from the 50 carcass rinse samples from the treated group analyzed in the laboratory. However, eight percent of 10 the rinse samples taken from the 50 carcasses from the control group were positive for Group C *Salmonella* indicating that the vaccine was efficacious in eliminating indigenous *Salmonella* from the meat product.

Table 14. Site #1 carcass rinse evaluation.

GROUP	NUMBER OF POSITIVE SAMPLES
Control	4/50 (8%)
Treated	0/50 (0%) <sup>1</sup>

<sup>1</sup> Significantly different from control group using Chi-square test ( $P < 0.05$ )

##### Trial at Site No. 2:

###### 1. Analyses of base-line samples.

Analyses of base-line samples collected revealed that the feed from the control house and the meconia from chick papers contained organisms suspected to be of O-antigen group C<sub>3</sub>, however pure isolates were not recovered. No other *Salmonella sp.* or vaccine organism could be identified from feed, water or from drag swabs of the litter from either house on the farm for the remainder of the 6-week grow-out period 25 of the trial.

###### 2. Livability.

Livability data were collected periodically for each house on the farm. Table 15 shows the mortality data during the grow-out period of the trial. Fewer birds expired in the control house than the treated house during the first week of the trial.

30 No differences in the number of birds that expired between the groups were observed

after the first week of the trial. The average percent mortality for a week during the trial period was 5.0 - 6.2 for the region of the country in which the trial took place (*The Poultry Informed Professional*, February 1998). The mortality in each house fell below the regional average for this period.

5

Table 15. Mortality of birds during the grow-out period for the Site #2 trial.

	CONTROLS	TREATED
Day 7	236	363
Day 14	88	73
Day 30	281	306
Day 35	64	60
Day 42	82	78
Total mortality headcount	751	880 <sup>1</sup>
Percent total	3.7	4.3

<sup>1</sup> Significantly different from control group using Chi-square test (P < 0.05)

10           The 0.6 percent difference in mortality between the treated and control birds in the first week is within the expected deviation observed for hatchlings. There was no difference in the numbers of mortality headcount after the first week through the end of the trial. This difference in first week mortality was not observed in two of the three trials conducted with the vaccine product. Factors that can affect survival of  
 15           hatchlings can include variations of the age of the breeder chickens, the quality of the breeder and hatchery management and extended exposure to temperatures less than 85°F. Survival percentiles for 40 - 42 day old birds previously raised on this test farm during the same year as the trial ranged from 94.7 to 97.5. The survival percentile for the control and treated birds fell within this range with 96.3 and 95.7 percent,  
 20           respectively.

### 3. FSIS Inspector's condemnation report at processing.

          The USDA Inspector's condemnation report for each house is presented in Table 16. Data for the average % for the same region of the country for site #2 was obtained from the *Poultry Informed Professional*, February, 1998. No difference in  
 25           the number of birds condemned in the control and treated groups was observed in this trial.

Table 16. Percent condemnations at Site #2 trial.

CONDEMNATION CAUSE	CONTROL	TREATED	AVERAGE % FOR SAME REGION AS SITE #2
Leukosis	<0.01	<0.01	0.01
Septicemia/Toxemia	0.21	0.13	0.41
Tumors	0.07	0.02	N/A <sup>1</sup>
Airsacculitis	0.25	0.18	0.39
Ascites	0.11	0.15	N/A
Inflammatory process	0.43	0.58	0.45
Overscald	0.05	0.01	N/A
Bruises	0.03	0.04	0.08
Tuberculosis	0	0	N/A
Cadavers	0.03	0.04	N/A
Contaminated	0.05	0.05	N/A
Dead on arrival	0.21	0.21	N/A
Total condemnations	1.44	1.41 <sup>2</sup>	

<sup>1</sup> N/A indicates data not available.

5       <sup>2</sup> Not significantly different from control group using Chi-square test at the P < 0.05 level.

#### 4. Evaluation of carcass weight at processing.

The average weight of birds during nine grow-out cycles on Site #2 ranged from 3.35 to 3.93 lbs; the treated bird weights fell within this range averaging 3.7 lbs  
10      at 42 days of age. The weight of the birds from the treated house were 6.1% lower than the control birds' average weight at processing. However, the weight of the treated birds cannot be compared to the control group as two of the four water lines in the treated house were discovered to be blocked during the fourth week of the trial. It was the opinion of the Broiler Manager that this weight difference may be partially or  
15      wholly due to this problem.

#### 5. Carcass rinse evaluation.

Table 17 shows *Salmonella* positive samples identified from carcass rinses in treatment group. No vaccine organisms or wild-type *Salmonella sp.* were identified in any of the 100 rinse samples from either group.

Table 17. Site #2 carcass rinse evaluation.

GROUP	NUMBER OF POSITIVE SAMPLES
Controls	0/50
Treated	0/50

**Trial at Site #3:**

## 5    1. Analyses of base-line samples.

Analyses of base-line samples collected revealed that the litter drag swabs taken from the control house contained organisms suspected to be of O-antigen Group C<sub>3</sub>, however a pure isolate could not be recovered. The chicks originating from two breeder flocks were culture negative for indigenous *Salmonella spp.* No other 10 *Salmonella spp.* or vaccine organism could be identified from feed, water or from drag swabs of the litter from either house on the farm for the remainder of the 64-day grow-out period of the trial.

2. Livability.

15    Livability data were collected weekly for each house. Table 18 shows the mortality data collected during the grow-out period of the trial.

Table 18. Mortality of birds during the grow-out period for Site #3 trial.

	CONTROLS	TREATED
1st Week	293	328
2nd Week	151	176
3rd Week	76	114
4th Week	60	70
5th Week	86	68
6th Week	103	97
7th Week	109	84
8th Week	97	118
9th Week	105	119
Total mortality headcount	1080	1174 <sup>1</sup>
Percent total	6.8	7.3

20    <sup>1</sup> Significantly different from control group using Chi-square test ( $P < 0.05$ )

It should be noted that a difference in mortality between the control and treated groups was due to only a five bird deviation. It is our speculation that a  
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standard deviation would exist due to the significant number of errors discovered on the daily mortality charts. Therefore, given the uncertainty of the actual headcounts one could expect a margin of error as low as 0.03% to contribute significantly to the final statistical differences. However, when the final survival values were compared 5 for the previous six-months grow-out cycles for this site for the year of the trial, percentiles ranged from 90 to 95. The livability percentiles for control and treated birds from this trial were 93.2 and 92.7, respectively, and fell within this range.

3. FSIS Inspector's condemnation report at processing.

Condemnation data for the two treatment groups from Site #3 carcasses was 10 inadvertently combined by the USDA Inspectors. The Inspector's condemnation report for both houses is presented in Table 19. Averages for the region including Site #3 are based on a 6-week old broiler for the same period as the trial (*The Poultry Informed Professional*, April, 1998). The condemnation rates for the present trial in the five categories specified in Table 19 are the same or lower than those for the 15 region of the country in which Site #3 was located as measured for a week during the trial period. The treatment of the birds with the vaccine did not adversely affect the condemnation percentiles in any category inspected.

Table 19. Percent condemnations at processing for Site #3.

CONDEMNATION CAUSE	COMBINED DATA (%) FOR TREATED AND CONTROL BIRDS	AVERAGE (%) FOR SAME REGION
Leukosis	<0.01	0.04
Septicemia/Toxemia	0.26	0.26
Tumors	0.01	N/A <sup>1</sup>
Airsacculitis	0.16	0.3
Ascites	0.05	N/A
Inflammatory process	0.1	0.3
Overscald	0	N/A
Bruises	0	0.01
Tuberculosis	0	N/A
Cadavers	0.04	N/A
Contaminated	<0.01	N/A
Dead on arrival	0.3	N/A
Total condemnations	0.92	

20 <sup>1</sup> N/A indicates data not available.

**4. Evaluation of carcass weight at processing.**

An examination of three previous grow-out cycles for Site #3 showed birds' weights ranging from 6.67 to 7.2 lbs. For this study, the average weight of the birds at processing was 7.33 lbs. It should be noted that although this producer has

5 experienced over the past year a decline in the quality of the chicks received from the breeder (as noted by the high mortality during the first two weeks of the trial), the excellent performance and final heavy weight of the birds at processing for this trial was unexpected. Based on these average weight data, the vaccine did not affect the ability of the birds to maintain a level of performance expected by the producer.

10 **5. Carcass rinse evaluation.**

Table 20 shows the results of the analyses of carcass rinse samples for each group. Neither the modified live *S. typhimurium* organisms nor any indigenous *Salmonella sp.* was recovered from the 50 treated house carcass rinse samples. However, twelve percent of the rinse samples taken from the 50 carcasses from the 15 control group were PCR and culture positive for *S. heidelberg* and *S. hadar*.

Table 20. Site #3 carcass rinse evaluation.

GROUP	NUMBER OF POSITIVE SAMPLES
Control	6/50 (12%) <sup>1</sup>
Treated	0/50 (0%) <sup>2</sup>

<sup>1</sup> Cultures were identified as either *S. heidelberg* or *S. hadar*.

20 <sup>2</sup> Significantly different from control group using Chi-square test ( $P < 0.05$ ).

**Conclusions:**

Spray vaccination with the live *S. typhimurium* vaccine,  $\chi$ 3985, U.S.D.A.

Product Code 19C1.01, was found to be safe for use in commercial broiler chickens.

25 The birds maintained a level of health and performance expected by the producer after exposure to the vaccine product. The immediate test environment was found to be free of vaccine residue. The vaccine was found to be efficacious in that its use resulted in complete elimination of indigenous *Salmonella sp.* on the carcasses from the treated groups compared to the control groups in two of three trials. These results

confirm that the vaccine was found to be both safe and efficacious in trials conducted in cooperation with three commercial poultry operations.

- All references cited in this specification, including without limitation all
- 5   patents, patent applications, reports, articles from journals, periodicals, textbooks and the like, manuscripts, internet website listings, brochures, and any and all other publications, are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by their authors and no admission is made that any reference constitutes prior art. Applicants reserve the  
10   right to challenge the accuracy and pertinency of the cited references.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results obtained.

- As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter  
15   contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.